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Rotenone-induced Impairment of Mitochondrial Electron Transport Chain Confers a Selective Priming Signal for NLRP3 Inflammasome Activation*

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Background: Mitochondrial dysfunction is considered crucial for triggering NLRP3 inflammasome activation.
Results: Rotenone-induced impairment of mitochondrial electron transport chain promotes NLRP3 inflammasome activation exclusively with ATP but not with other NLRP3-activating stimulators.
Conclusion: High-grade mROS and hyperpolarization are essential for NLRP3 inflammasome activation upon rotenone-induced mitochondrial dysfunction.
Significance: Mitochondrial impairment may selectively prime NLRP3 inflammasome activation, leading to many degenerative diseases.

Mitochondrial dysfunction is considered crucial for NLRP3 inflammasome activation partly through its release of mitochondrial toxic products, such as mitochondrial reactive oxygen species (mROS)[2] and mitochondrial DNA (mtDNA). Although previous studies have shown that classical NLRP3-activating stimulations lead to mROS generation and mtDNA release, it remains poorly understood whether and how mitochondrial damage-derived factors may contribute to NLRP3 inflammasome activation. Here, we demonstrate that impairment of the mitochondrial electron transport chain by rotenone primes NLRP3 inflammasome activation only upon costimulation with ATP and not with nigericin or alum. Rotenone-induced priming of NLRP3 in the presence of ATP triggered the formation of specklike NLRP3 or ASC aggregates and the association of NLRP3 with ASC, resulting in NLRP3-dependent caspase-1 activation. Mechanistically, rotenone confers a priming signal for NLRP3 inflammasome activation only in the context of aberrant high-grade, but not low-grade, mROS production and mitochondrial hyperpolarization. By contrast, rotenone/ATP-mediated mtDNA release and mitochondrial depolarization are likely to be merely an indication of mitochondrial damage rather than triggering factors for NLRP3 inflammasome activation. Our results provide a molecular insight into the selective contribution made by mitochondrial dysfunction to the NLRP3 inflammasome pathway.

The inflammasome is a multiprotein complex that is assembled upon sensing of pathogen- or danger-associated molecular patterns by a sensor molecule, such as nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 (NLRP3) (1). The assembled inflammasome complex promotes the activation of caspase-1, leading to the processing and release of interleukin-1β (IL-1β) (2). Activation of inflammasome signaling thus triggers the initial inflammatory responses at infected or injured sites primarily by secreting IL-1β; however, deregulated or chronic inflammasome activation is potentially implicated in metabolic and neurodegenerative disorders, such as type 2 diabetes and Alzheimer disease (3, 4).

Although NLRP3 is the best characterized inflammasome sensor molecule, its detailed activation mechanism remains poorly understood (5). Conventionally, the activation and assembly of the NLRP3 inflammasome require two independent signals: signal 1 for transcriptional induction of NLRP3 and pro-IL-1β and signal 2 for the activation of inflammasome components (2). However, recent findings have revealed that the post-transcriptional modification of NLRP3 by either signal 1 or signal 2 is also crucial for NLRP3 inflammasome activation (6–8). The way in which the NLRP3 inflammasome is assembled and activated in response to a wide range of stimulations is therefore now considered to be more complicated than previously believed. Much attention is now being paid to the potential role played by mitochondria in modulating NLRP3 inflammasome activation as a central common hub for diverse agonists (9). Recent evidence has shown that NLRP3-activating stimulations induce mitochondrial damage, resulting in the release of mitochondrial reactive oxygen species (mROS) and mitochondrial DNA (mtDNA), and it has been hypothesized that these mitochondria-derived toxic products may act as crucial mediators in activating NLRP3 (10–13).

Mitochondrial dysfunction has been extensively studied in terms of its connection to multiple degenerative disorders (14, 15). Considering that NLRP3 inflammasome signaling is also potentially implicated in metabolic or neurodegenerative dis-

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[2] The abbreviations used are: mROS, mitochondrial reactive oxygen species; mtDNA, mitochondrial DNA; BMDM, bone marrow-derived macrophage; ETC, electron transport chain; MPT, mitochondrial permeability transition; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NAC, *N*-acetyl-L-cysteine; Z-, benzyloxycarbonyl-; fmk, fluoromethyl ketone.

orders, mitochondrial dysfunction may contribute to the pathogenesis of degenerative diseases by activating the NLRP3 inflammasome. Mitochondrial dysfunction may be experimentally induced by several chemicals, including rotenone (16). Rotenone impairs mitochondrial respiration by inhibiting electron transport chain (ETC) complex I, leading to ATP depletion, mROS production, and the loss of mitochondrial membrane potential (16, 17). Interestingly, rotenone is also used to induce Parkinson disease in experimental animal models, possibly through the malfunction of ETC complex I (18).

Previous studies have shown that the inhibition of mitochondrial ETC complex by rotenone or antimycin A leads to NLRP3 inflammasome activation (10). By contrast, various studies have demonstrated that rotenone fails to activate caspase-1 (19) or even suppresses caspase-1 activation upon NLRP3-dependent stimuli, such as ATP, nigericin, alum, or silica (13). Similarly, a protonophore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which induces the dissipation of mitochondrial membrane potential and mitochondrial fission, was reported to promote or inhibit NLRP3 inflammasome activation (10, 20). In this regard, the contribution made by mitochondrial impairment to the NLRP3 pathway requires additional clarification in order to provide a molecular insight into the mechanism underlying NLRP3 inflammasome activation. We therefore closely examined how direct mitochondrial dysfunction affects NLRP3 inflammasome activation.

Experimental Procedures

Reagents and Antibodies—LPS, rotenone, CCCP, ATP, nigericin, *N*-acetyl-L-cysteine (NAC), cyclosporin A, menadione, and U0126 were purchased from Sigma-Aldrich. Alum was obtained from InvivoGen. Z-VAD-fmk was obtained from Bachem. Mito-SOX, MitoTracker Green, and MitoTracker Deep Red were purchased from Invitrogen. Mito-TEMPO was purchased from Santa Cruz Biotechnology, Inc. PR619 was purchased from LifeSensors. Anti-IL-1β antibody (AF-401-NA) was obtained from R&D Systems. Anti-caspase-1 (AG-20B-0042) and NLRP3 (AG-20B-0014) antibodies were purchased from Adipogen. Anti-ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) antibody (SC-22514) was purchased from Santa Cruz Biotechnology.

Cell Culture—Mouse bone marrow-derived macrophages (BMDMs) were prepared from C57BL/6 or *Nlrp3*-deficient mice as described previously (21). All mice were maintained in specific pathogen-free conditions. Protocols for mice experiments were approved by the Institutional Ethical Committee of the Yonsei University College of Medicine. Immortalized wild-type, *Asc*-deficient, *Nlrp3*-deficient, NLRP3-reconstituted (N1–8), or NLRP3-GFP-expressing mouse BMDMs were kindly provided by Dr. E. S. Alnemri (Thomas Jefferson University, Philadelphia, PA). All of the BMDMs were grown in L929-conditioned DMEM, which was supplemented with 10% FBS and 100 units/ml penicillin-streptomycin.

Immunoblot Analysis—Cells were lysed in 20 mM HEPES (pH 7.5) buffer containing 0.5% Nonidet P-40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and protease inhibitors. Soluble lysates underwent SDS-PAGE and were then immunoblotted with the appropriate antibodies. For the coimmunoprecipitation experiment, cells were lysed in 10 mM HEPES buffer (pH 7.4) containing 0.2% Nonidet P-40, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, and protease inhibitors. Lysates were precleared with Protein G-Sepharose 4 beads and immunoprecipitated with the anti-ASC antibody, and the bead-bound proteins were immunoblotted with the appropriate antibodies. All of the blots were representative images of at least three independent experiments.

Quantification of mRNA Expression—To measure mRNA expression of *IL-1β*, *Nlrp3*, and *Asc*, a quantitative real-time PCR assay was performed as described previously (22). Primers were as follows: 5′-GCC CAT CCT CTG TGA CTC AT-3′ and 5′-AGG CCA CAG GTA TTT TGT CG-3′ (*IL-1β*); 5′-ATG CTG CTT CGA CAT CTC CT-3′ and 5′-AAC CAA TGC GAG ATC CTG AC-3′ (*Nlrp3*); and 5′-CTT GTC AGG GGA TGA ACT CAA AA-3′ and 5′-GCC ATA CGA CTC CAG ATA GTA GC-3′ (*Asc*).

Assay of Inflammasome Activation and Assembly—To activate NLRP3 inflammasome signaling, BMDMs were primed with LPS (0.25 μg/ml) for 2–3 h, followed by treatment with ATP (2.5 mM, 30 min), nigericin (5 μM, 45 min), or alum (250 μg/ml, 6 h). To determine inflammasome activity, the culture supernatants were precipitated as described previously and then immunoblotted with anti-caspase-1 or IL-1β antibody (23). To determine the oligomerization of NLRP3, confocal microscopy of NLRP3-GFP-expressing BMDMs was performed using a confocal microscope (Zeiss LSM 700), as described previously (22). To determine the oligomerization of ASC, a chemical cross-linking assay was performed using disuccinimidyl suberate as described previously (24). To visualize ASC specklike oligomeric structure, an immunofluorescence assay was performed using anti-ASC antibody as described previously (22).

Assay of mROS, mtDNA Release, and Mitochondrial Membrane Potential—To measure mROS production, cells were resuspended in Hanks' balanced salt solution after appropriate treatments and stained with MitoSOX (2.5 μM) at 37 °C for 20 min. The fluorescence of the cells was monitored and analyzed by a flow cytometer (FACSVerse, BD Biosciences). To determine cytosolic mtDNA, cells were lysed, and cytosolic fractions were prepared as described previously (11). Next, DNA was isolated from the normalized cytosolic fraction, and the copy number of DNA encoding cytochrome *c* oxidase I was determined via quantitative real-time PCR using the following primers: 5′-GCC CCA GAT ATA GCA TTC CC-3′ (forward) and 5′-GTT CAT CCT GTT CCT GCT CC-3′ (reverse). To determine mitochondrial potential-dependent damage, cells were costained with MitoTracker Deep Red and MitoTracker Green according to the manufacturer's protocol. Cells were then analyzed by a flow cytometer.

Transmission Electron Microscopy—Cells were fixed with 2% glutaraldehyde-paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h. After washing, cells were postfixed with 1% OsO_4 in 0.1 M phosphate buffer for 2 h and dehydrated in ascending gradual series (50–100%) of ethanol. Specimens were embedded using a Poly/Bed 812 kit (Polysciences, Inc.). 70-nm thin sections were stained with uranyl acetate and lead

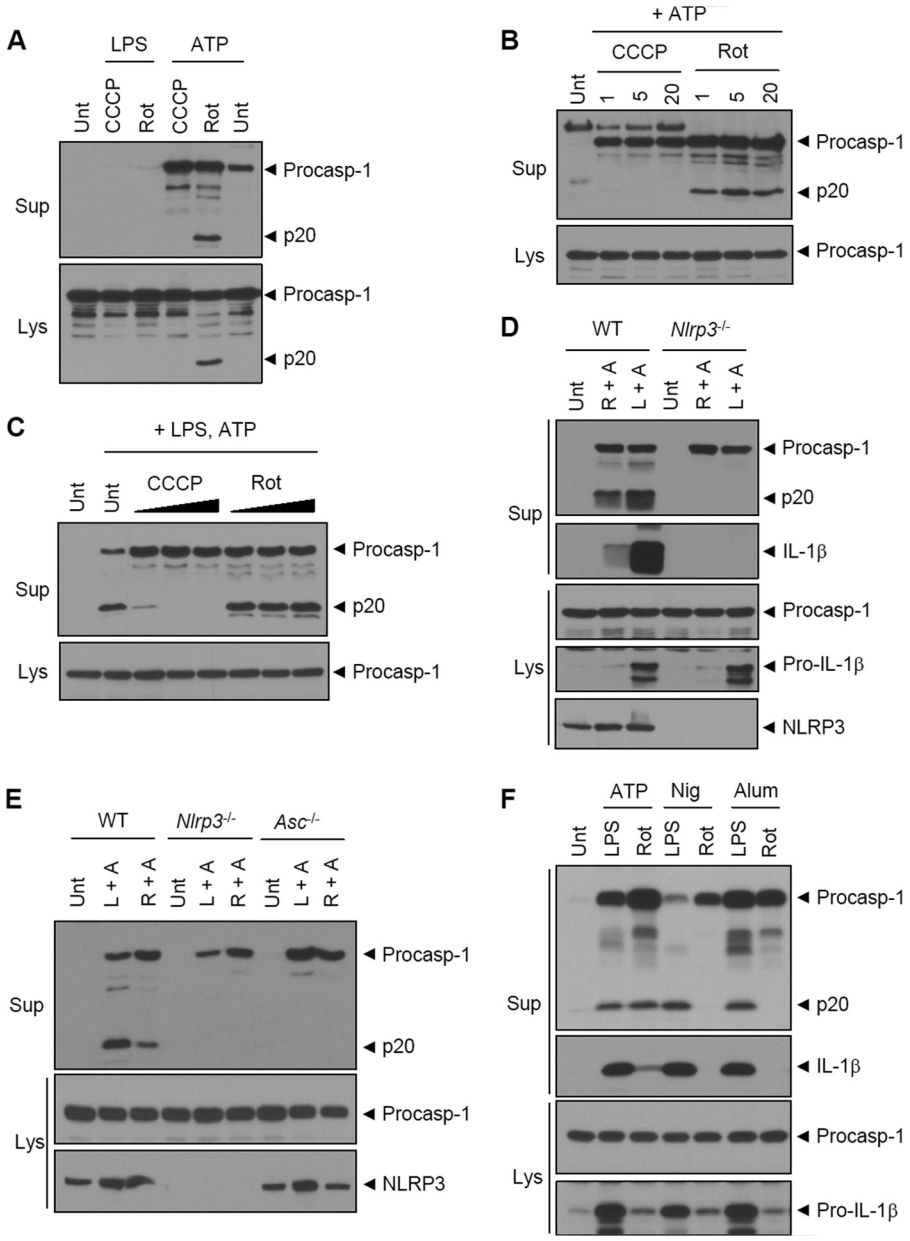

FIGURE 1. **Rotenone promotes NLRP3 inflammasome activation only with ATP costimulation.** *A*, mouse BMDMs were untreated (*Unt*) or treated with LPS (0.25 μg/ml) for 3 h, followed by treatment with CCCP (40 μM) or rotenone (*Rot*) (10 μM) for 2 h, or were treated with CCCP or rotenone for 2 h, followed by treatment with ATP (2.5 mM, 30 min), as indicated. *B*, mouse BMDMs were treated with CCCP or rotenone (1–20 μM) for 2 h, followed by treatment with ATP. *C*, mouse BMDMs were treated with LPS in the presence of CCCP or rotenone (1–10 μM) for 2 h, followed by ATP treatment. *D* and *E*, primary WT or *Nlrp3*$^{-/-}$ BMDMs (*D*), or immortalized WT, *Nlrp3*$^{-/-}$, or *Asc*$^{-/-}$ BMDMs (*E*) were untreated or treated with LPS or rotenone (10 μM) for 3 h, followed by treatment with ATP. *F*, mouse BMDMs were treated with LPS or rotenone (5 μM) for 3 h, followed by ATP, nigericin (*Nig*), or alum treatment, as indicated under "Experimental Procedures." *A–F*, culture supernatants (*Sup*) or cellular lysates (*Lys*) were immunoblotted with the indicated antibodies.

citrate. Stained sections were then observed using a JEM-1011 (JEOL) transmission electron microscope.

Statistical Analysis—All values are expressed as the mean and S.E. of individual samples. Data were analyzed using Student's *t* test. *p* values of ≤0.05 were considered significant.

Results

Rotenone Induces NLRP3-dependent Caspase-1 Activation with ATP but Not with Other NLRP3 Stimulators—To provide a molecular insight into how mitochondrial dysfunction is implicated in the activation of NLRP3 inflammasome signaling, we first determined whether the mitochondrial impairment-inducing chemicals rotenone and CCCP could function as a signal 2 stimulus for caspase-1 activation. Contrary to expectations, stimulation with rotenone or CCCP failed to trigger caspase-1 activation in LPS-primed BMDMs (Fig. 1*A*). Of particular note was the fact that rotenone, but not CCCP, promoted a robust caspase-1 processing with costimulation of ATP in BMDMs (Fig. 1, *A* and *B*). Conversely, CCCP attenuated LPS/ATP-triggered caspase-1 activation (Fig. 1*C*). Like LPS/ATP stimulation, rotenone/ATP stimulation caused an NLRP3- and ASC-dependent caspase-1 activation (Fig. 1, *D* and

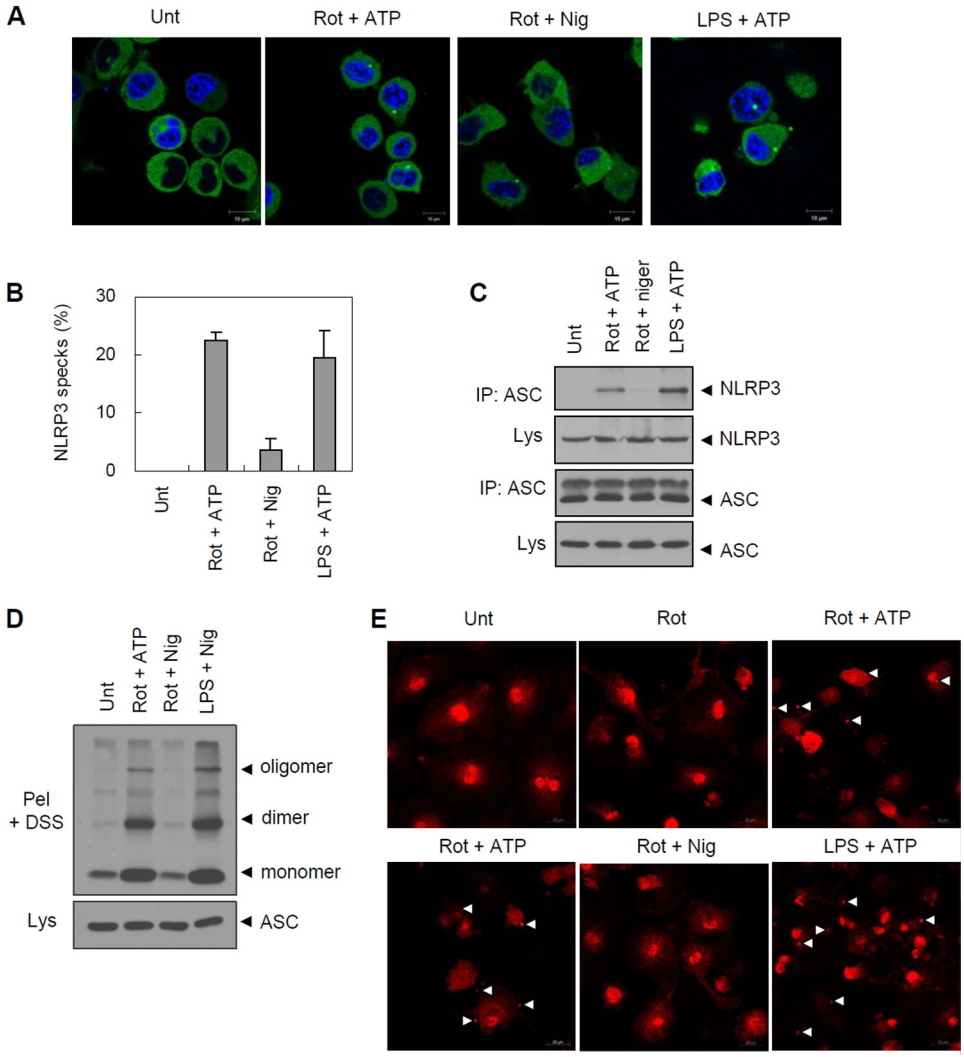

FIGURE 2. **Rotenone promotes the formation of NLRP3 inflammasome assembly only with ATP costimulation.** *A* and *B*, immortalized NLRP3-GFP-expressing BMDMs were treated with rotenone (*Rot*) (5 μM) or LPS (0.25 μg/ml) for 3 h, followed by ATP (2.5 mM, 30 min) or nigericin (*Nig*) (5 μM, 40 min) treatment. Specklike aggregates of NLRP3 were observed by confocal microscope (*A*, *scale bars*, 10 μm), and the relative percentages of NLRP3 speck-containing cells were plotted (*B*; $n = 3$). *C*, mouse BMDMs were treated with rotenone (5 μM) or LPS (0.5 μg/ml) for 2 h, followed by treatment with ATP (2 mM) or nigericin (5 μM) for 30 min. Soluble lysates were immunoprecipitated (*IP*) with anti-ASC antibody, and the immunoprecipitates or soluble lysates were immunoblotted, as indicated. *D*, mouse BMDMs were treated with LPS or rotenone (5 μM) for 3 h, followed by ATP or nigericin treatment. ASC oligomerization was assayed as described under "Experimental Procedures." *E*, mouse BMDMs were treated with rotenone or LPS, followed by ATP or nigericin treatment. Cells were stained with anti-ASC antibody and observed by confocal microscope. *Scale bars*, 20 μm. *Error bars*, S.E.

E), suggesting that rotenone appears to function as a signal 1 priming stimulus for NLRP3 inflammasome activation. However, rotenone had a negligible effect on transcriptional induction of pro-IL-1β and thus induced much lower IL-1β secretion with ATP costimulation as compared with LPS (Fig. 1*D*). These results indicate that the impairment of mitochondrial complex I by rotenone may facilitate the activation of NLRP3 inflammasome in a transcription-independent manner.

Next, we tested the NLRP3-priming role played by rotenone with costimulation of other well known NLRP3 stimuli. Intriguingly, rotenone did not promote any caspase-1 activation upon costimulation with nigericin or alum (Fig. 1*F*). This unexpected finding indicates that rotenone-induced mitochondrial dysfunction may mediate selective NLRP3 inflammasome activation exclusively with costimulation of ATP, but not of other conventional activators.

Rotenone Promotes the Assembly of NLRP3 Inflammasome Only in the Presence of ATP—To examine whether rotenone-triggered mitochondrial dysfunction mediates the assembly of NLRP3 inflammasome, we determined the formation of speck-like aggregates of NLRP3 or ASC and examined the association between NLRP3 and ASC. Both molecular assemblies are essential prerequisite steps for the activation of NLRP3 inflammasome (25, 26). Consistent with the aforementioned results, rotenone with ATP costimulation, but not with nigericin costimulation, induced a robust formation of NLRP3 specklike aggregates (Fig. 2, *A* and *B*). Rotenone also promoted substantial association of NLRP3 with ASC exclusively in the presence of ATP costimulation and not in the presence of nigericin (Fig. 2*C*). Supporting these data, rotenone/ATP costimulation, but not rotenone/nigericin, triggered a robust ASC oligomerization and ASC speck formation (Fig. 2, *D* and *E*). These observations

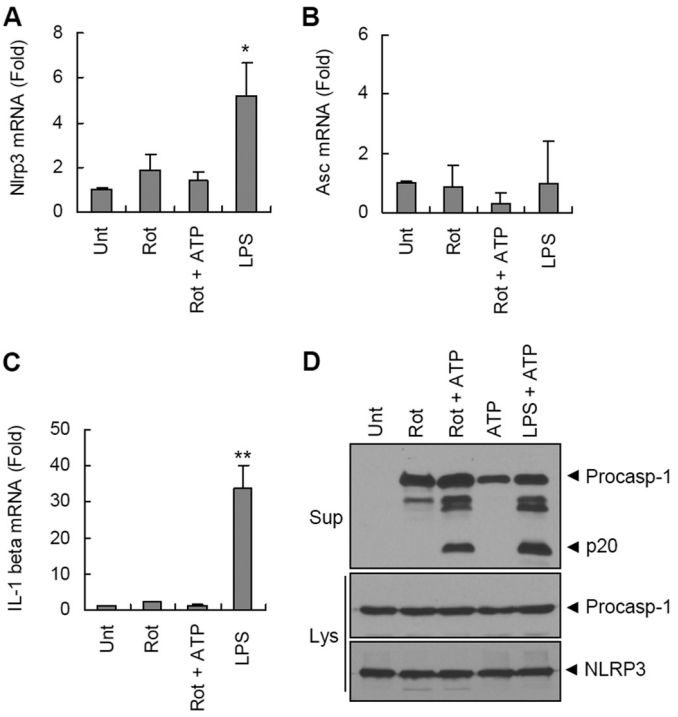

FIGURE 3. **Rotenone/ATP stimulation causes transcription-independent caspase-1 activation.** *A–C*, mouse BMDMs were treated with rotenone (*Rot*) or LPS, followed by ATP treatment, as indicated. Then mRNA levels of *Nlrp3*, *Asc*, or *IL-1β* were determined by quantitative real-time PCR. *Asterisks* indicate significant difference as compared with untreated (*Unt*) samples (*, $p < 0.05$, $n = 3$ (*A*); **, $p < 0.01$, $n = 3$ (*C*)). *D*, immortalized NLRP3-reconstituted BMDMs (N1–8) were treated with rotenone or LPS, followed by ATP treatment. Culture supernatants (*Sup*) or cellular lysates (*Lys*) were immunoblotted with the indicated antibodies. *Error bars*, S.E.

also support the contention that rotenone-mediated mitochondrial dysfunction may selectively prime the assembly of NLRP3 inflammasome only in the context of specific costimulation, such as ATP costimulation.

Rotenone/ATP Stimulation Promotes the Transcription-independent Caspase-1 Activation—To examine whether rotenone-mediated priming of NLRP3 inflammasome activation involves the transcription of NLRP3 or ASC, we measured mRNA levels of NLRP3 and ASC. Neither rotenone nor rotenone/ATP stimulation induced a significant increase in the transcription level of NLRP3, ASC, or pro-IL-1β (Fig. 3, *A–C*). To further verify whether rotenone/ATP stimulation could activate NLRP3 inflammasome in a transcription-independent manner, we performed a similar experiment in the reconstituted NLRP3-expressing macrophages (N1–8), which stably express NLRP3 irrespective of Toll-like receptor signaling, as reportedly previously (6). As observed in primary or immortalized BMDMs, rotenone induced a robust caspase-1 activation only with ATP costimulation (Fig. 3*D*).

Rotenone/ATP Stimulation Induces High-grade mROS Production and mtDNA Release into the Cytosol—Previous studies have demonstrated that inhibition of the mitochondrial electron transport chain by rotenone or antimycin A leads to the generation of mROS (10, 17). To test whether rotenone-mediated mROS production is responsible for NLRP3 inflammasome activation upon rotenone/ATP stimulation, we determined the intracellular level of mROS using MitoSOX staining. Rotenone treatment substantially increased mROS-producing macrophages, but the level of mROS production was low in most MitoSOX-positive cells (Fig. 4, *A* and *B*). Indeed, the population of high-grade mROS-producing macrophages was only slightly increased by rotenone (5.58%; Fig. 4*A*). Conversely, ATP stimulation caused a considerable increase (25–27%) in strong mROS-producing cells (Fig. 4, *A* and *B*). Considering that stimulation with ATP alone did not induce caspase-1 activation (Figs. 1*A* and 3*D*), ATP-induced high-grade mROS production is not sufficient for caspase-1 activation. Intriguingly, rotenone/ATP stimulation caused a remarkable increase in strong mROS-positive cells, whereas rotenone/nigericin treatment showed no such increase (Fig. 4, *A* and *B*). This finding suggests that highly elevated strong mROS production by rotenone/ATP may contribute to caspase-1 activation. Similarly, rotenone/ATP stimulation promoted a marked release of mtDNA into the cytosol, whereas rotenone or rotenone/nigericin induced much less cytosolic release of mtDNA (Fig. 4*C*). These data collectively indicate that rotenone/ATP stimulation, but not rotenone/nigericin stimulation, mediates the production of strong mROS and the cytosolic release of mtDNA, possibly contributing to NLRP3 inflammasome activation.

Rotenone/ATP-induced Generation of Mitochondrial Toxic Products Differentially Contributes to NLRP3 Inflammasome Activation—A recent study has proposed that active caspase-1 induces mitochondrial damage as an effector function of caspase-1 (27). In order to examine whether the increased strong mROS production and mtDNA release are consequences of augmented caspase-1 activation by rotenone/ATP stimulation, we assessed the rotenone/ATP-triggered generation of mitochondrial toxic products in inflammasome activity-deficient $Asc^{-/-}$ BMDMs. As observed in wild-type BMDMs, rotenone/ATP stimulation induced a marked increase in strong mROS production in $Asc^{-/-}$ BMDMs (Fig. 5*A*). Supporting these data, pan-caspase inhibitor Z-VAD did not impair rotenone/ATP-triggered strong mROS production (Fig. 5*B*), demonstrating that the elevated strong mROS production is not caused by enhanced caspase-1 activation. Furthermore, the antioxidant NAC efficiently abolished rotenone/ATP-triggered caspase-1 activation (Fig. 5*C*). These results indicate that high-grade mROS production is crucial for rotenone/ATP-mediated caspase-1 activation. By contrast, CCCP did not induce the production of high-grade mROS (Fig. 5*D*). This might partly explain the failure of CCCP to activate caspase-1 (Fig. 1, *A* and *B*), contrary to the previous report (10).

Unlike mROS production, mtDNA release by rotenone/ATP was significantly impaired in $Asc^{-/-}$ BMDMs (Fig. 5*E*). Moreover, the inhibition of caspase-1 activation by Z-VAD similarly decreased the rotenone/ATP-induced mtDNA release into the cytosol (Fig. 5*F*). These findings suggest that some indications of mitochondrial damage, such as mtDNA release, may reflect potentiated caspase-1 activation by rotenone/ATP stimulation. Furthermore, cyclosporin A was previously shown to inhibit NLRP3-mediated caspase-1 activation by blocking mitochondrial membrane permeability transition (MPT) pores (13). We therefore examined whether the MPT is implicated in rotenone/ATP-mediated caspase-1 activation. Cyclosporin A largely abolished LPS/ATP-mediated caspase-1 activation but

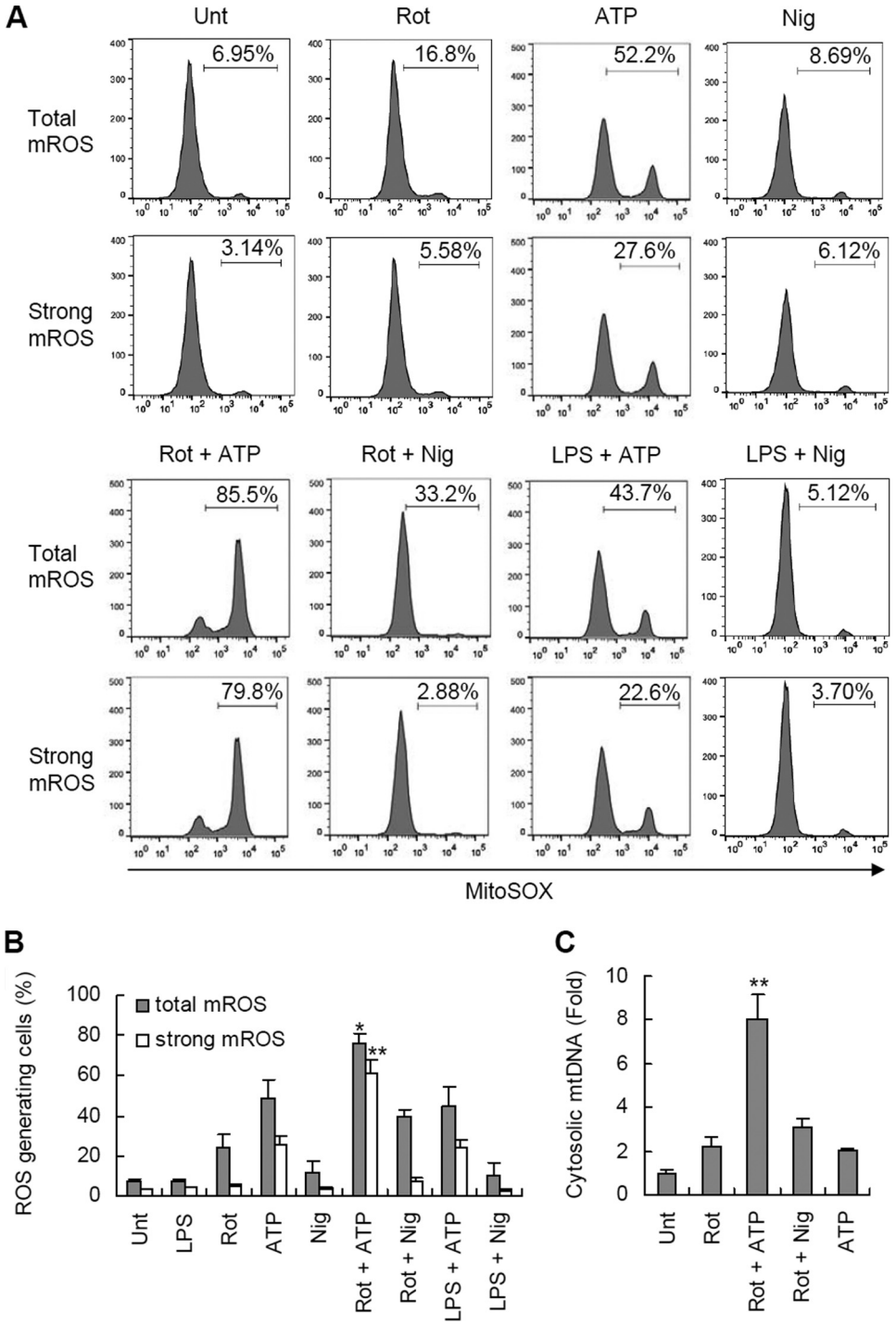

FIGURE 4. **Rotenone/ATP stimulation causes strong mROS production and mtDNA release into the cytosol.** *A* and *B*, mouse BMDMs were untreated (*Unt*) or treated with rotenone (*Rot*) (5 μM) or LPS for 2 h, followed by treatment with ATP (2.5 mM, 20 min) or nigericin (*Nig*) (5 μM, 30 min), as indicated. The cells were then stained with MitoSOX and analyzed by a flow cytometer. Four or three independent experiments were performed, and total mROS- or strong mROS-generating cells are plotted in *B*. *Asterisks* indicate significant differences as compared with ATP-treated samples ($n = 4$; *, $p < 0.05$; **, $p < 0.005$). *C*, mouse BMDMs were treated with rotenone (5 μM, 3 h), followed by treatment with ATP (2.5 mM, 20 min) or nigericin (5 μM, 30 min). Cytosolic mtDNA was quantified as described under "Experimental Procedures." *Asterisks* indicate significant differences as compared with rotenone-treated samples ($n = 9$; **, $p < 0.0005$). *Error bars*, S.E.

not rotenone/ATP-induced caspase-1 activation (Fig. 5*G*). This finding indicates that rotenone/ATP-mediated caspase-1 activation is independent of MPT.

mROS Production Is Not Sufficient to Promote NLRP3 Inflammasome Activation—To provide more insight into the rotenone/ATP-mediated caspase-1 activation, we observed mitochondrial morphology by transmission electron microscopy. Rotenone/ATP costimulation as well as treatment with ATP alone induced a disruption of mitochondrial cristae, leading to the formation of vesicular matrix compartments (Fig. 6*A*). The disruption of mitochondrial crista organization was inhibited by Mito-TEMPO, a specific mROS scavenger, but not

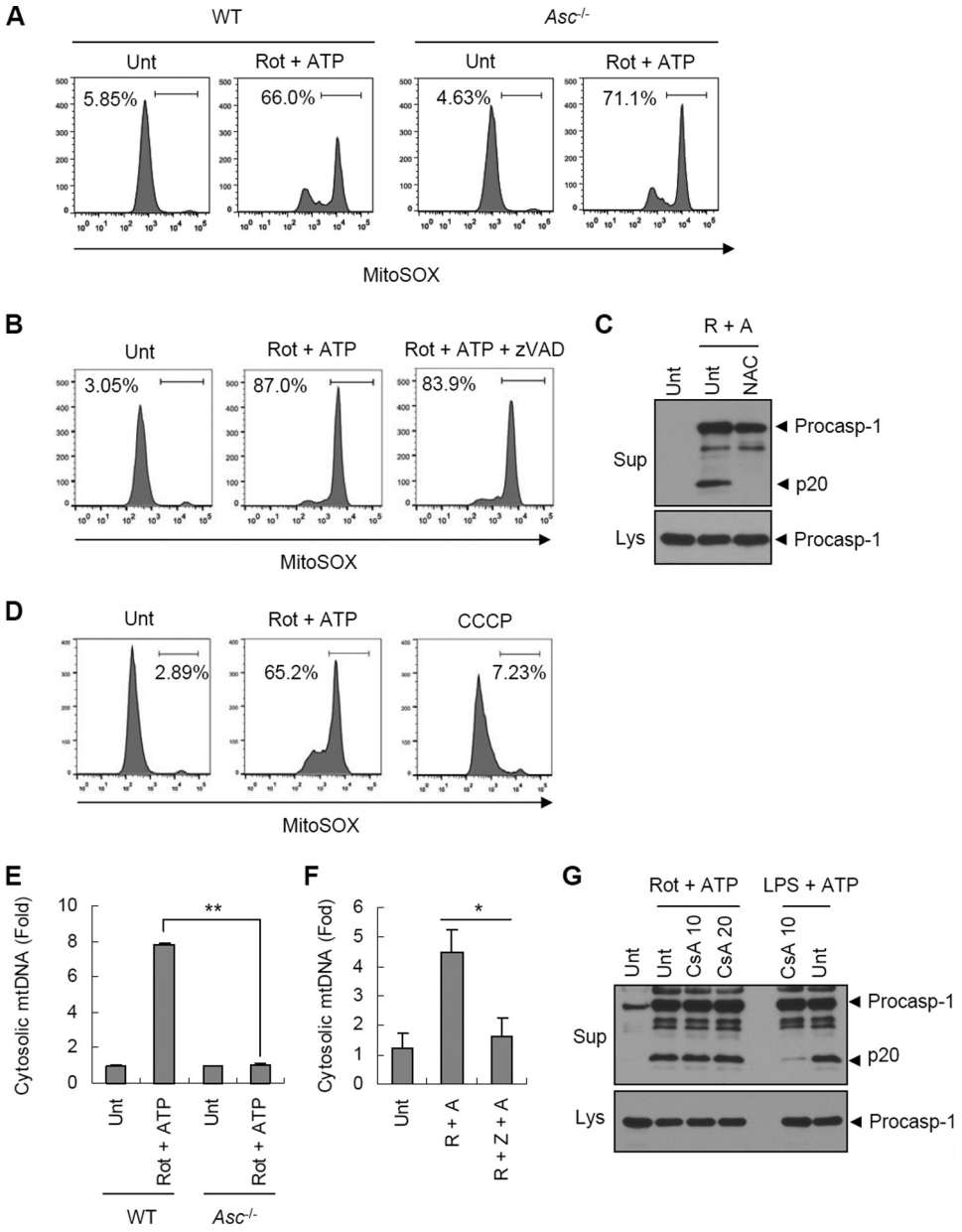

FIGURE 5. **Inflammasome activation is differentially responsible for rotenone/ATP-induced mROS production and mtDNA release.** *A* and *B*, immortalized WT or $Asc^{-/-}$ BMDMs (*A*) or primary WT BMDMs (*B*) were treated with rotenone (*Rot*) (5 μM, 2 h) in the presence of Z-VAD-fmk (20 μM) (*B*), followed by treatment with ATP (2.5 mM, 15 min (*A*) or 20 min (*B*)). *C*, mouse BMDMs were treated with rotenone (5 μM, 3 h) in the presence of NAC (20 mM), followed by treatment with ATP. *D*, mouse BMDMs were treated with rotenone, followed by treatment with ATP, or treated with CCCP (20 μM, 2 h). *A*, *B*, and *D*, mROS production was assayed using MitoSOX staining as in Fig. 4. *E* and *F*, immortalized WT or $Asc^{-/-}$ BMDMs (*E*) or primary BMDMs (*F*) were treated with rotenone in the presence of Z-VAD-fmk (20 μM) (*F*), followed by treatment with ATP. Cytosolic mtDNA was assayed, and *asterisks* indicate significant differences ($n = 3$; *, $p < 0.05$; **, $p < 0.0001$). *G*, mouse BMDMs were treated with rotenone or LPS (3 h) in the presence of cyclosporin A (*CsA*; 10 or 20 μM), followed by treatment with ATP. *C* and *G*, culture supernatants or soluble lysates were immunoblotted. *Unt*, untreated. *Error bars*, S.E.

by Z-VAD. This finding proposed that high-grade mROS production by rotenone/ATP or ATP is critical for the deranged cristae. However, mROS-mediated mitochondrial dysfunction is not sufficient for caspase-1 activation, because ATP failed to activate caspase-1.

To verify whether mROS production could mediate NLRP3 inflammasome activation, BMDMs were treated with the ROS generator menadione. As reported previously (28), menadione promoted considerable mROS production (Fig. 6*B*). Of note, menadione/ATP induced robust high-grade mROS production, similar to rotenone/ATP (Fig. 6*B*). However, menadione stimulation did not cause caspase-1 activation in the presence of LPS or ATP (Fig. 6*C*), indicating that other crucial factors in addition to mROS are required for the activation of NLRP3 inflammasome upon mitochondrial dysfunction.

Mitochondrial Hyperpolarization Is Important in Rotenone/ATP-mediated Caspase-1 Activation—Mitochondrial membrane potential is not only essential for producing cellular energy but is also required for regulating innate immune responses (20, 29). We therefore examined whether alterations in mitochondrial membrane potential are implicated in the activation of NLRP3 inflammasome upon rotenone/ATP stim-

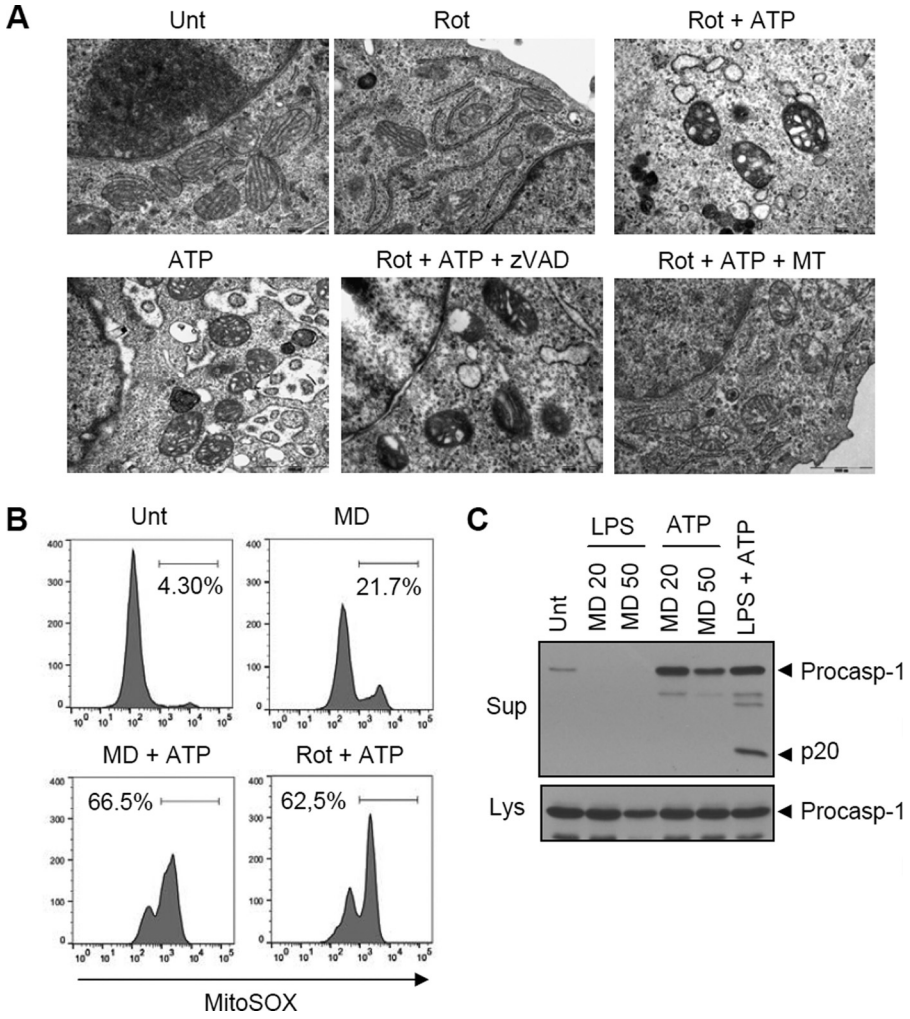

FIGURE 6. **Strong mROS production impairs mitochondrial structure but is not sufficient to activate NLRP3 inflammasome.** *A*, transmission electron microscopy images of mouse BMDMs treated with rotenone (*Rot*) (5 μM, 3 h) in the presence of Z-VAD-fmk (20 μM) or Mito-TEMPO (*MT*) (250 μM), followed by ATP treatments (2.5 mM, 10 min), as indicated. *Scale bars*, 1 μm. *B*, mouse BMDMs were treated with menadione (*MD*) (20 μM) or rotenone (5 μM) for 2 h, followed by ATP treatments (2.5 mM, 15 min). Cells were then stained with MitoSOX and analyzed by a flow cytometer. *C*, mouse BMDMs were treated with LPS (0.25 μg/ml, 3 h), followed by menadione (2 h) or ATP treatment, or treated with menadione (2 h), followed by ATP treatments, as indicated. Cultural supernatants or cellular lysates were immunoblotted with anti-caspase-1 antibody. *Unt*, untreated.

ulation. Rotenone treatment exhibited a slight increase in mitochondrial membrane potential, as determined by costaining of Mitotracker Deep Red and Mitotracker Green (7.3%) (Fig. 7*A*). The fluorescence intensity of Mitotracker Deep Red represents membrane potential. Of greater interest, ATP stimulation in the presence of rotenone pretreatment caused a dramatic increase in the cell population containing hyperpolarized mitochondria (Rot + ATP; Fig. 7*A*), whereas nigericin in the presence of rotenone exhibited a negligible effect. In addition, the population of cells containing damaged or depolarized mitochondria (*bottom right box*) was also increased only in rotenone/ATP-stimulated BMDMs (Fig. 7*A*). These results clearly indicate that rotenone/ATP stimulation causes a marked alteration in mitochondrial membrane potential that may modulate the activation of NLRP3 inflammasome.

Interestingly, the cells containing depolarized mitochondria upon rotenone/ATP stimulation showed a clear reduction in cell size, as determined by forward scatter signature, whereas the cells containing hyperpolarized mitochondria exhibited a slight increase in cell size and complexity as compared with untreated cells (Fig. 7*B*). Furthermore, the population of cells containing depolarized mitochondria increased along with ATP treatment time, whereas that of hyperpolarized mitochondria-containing cells decreased (Fig. 7*C*). Thus, mitochondrial depolarization may reflect mitochondrial damage rather than acting as the causative factor in NLRP3 inflammasome activation.

Indeed, a recent study has shown that the LPS/ATP-mediated increase in mitochondrial damage is dependent on caspase-1 activity (27). To determine whether the aforementioned hyperpolarization or depolarization of mitochondrial membrane potential is a consequence of inflammasome activation, we examined the rotenone/ATP-induced alteration of mitochondrial membrane potential in ASC-deficient macrophages. Consequently, mitochondrial depolarization as well as hyperpolarization were also evident in $Asc^{-/-}$ BMDMs upon rotenone/ATP stimulation, as in wild-type BMDMs (Fig. 8, *A* and *B*). These data suggest that rotenone/ATP-induced alterations in mitochondrial membrane potential did not result from caspase-1/inflammasome activation. Furthermore, depolarized

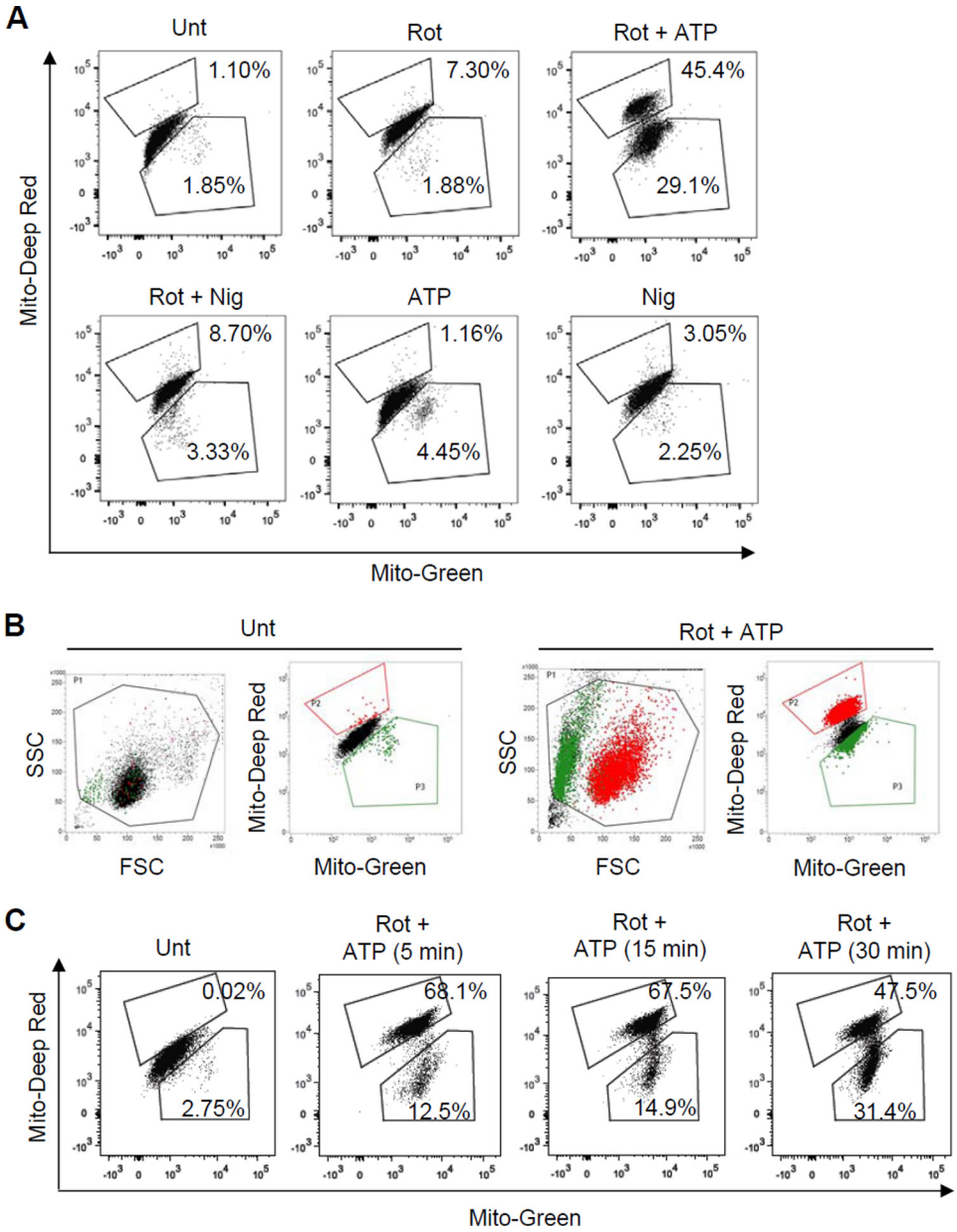

FIGURE 7. **Rotenone/ATP stimulation causes alterations in mitochondrial membrane potential.** *A* and *B*, mouse BMDMs were treated with rotenone (*Rot*), followed by treatment with ATP (2.5 mM, 20 min) or nigericin (*Nig*) (5 μM, 30 min). Cells were costained with MitoTracker Deep Red and MitoTracker Green and analyzed by a flow cytometer. Cells at the *top left* and the *bottom right* represent mitochondrial hyperpolarization and depolarization, respectively. Cells were also analyzed by forward scatter (*FSC*) and side scatter (*SSC*) in *B*. *C*, mouse BMDMs were treated with rotenone, followed by ATP (5, 15, or 30 min), as indicated. Cells were stained and analyzed by a flow cytometer as in *A*. *Unt*, untreated.

mitochondria-containing cells in *Asc*$^{-/-}$ macrophages also exhibited reduced cell size (Fig. 8*C*), demonstrating that rotenone/ATP-induced mitochondrial damage is inflammasome activity-independent. More importantly, mitochondrial depolarization by CCCP markedly abolished rotenone/ATP-mediated caspase-1 activation (Fig. 8*D*). Therefore, we concluded that mitochondrial hyperpolarization is critical for rotenone/ATP-mediated triggering of NLRP3 inflammasome activation, but depolarization is an indication of mitochondrial damage.

As described above, a well known mROS generator, menadione, failed to activate NLRP3 inflammasome activation. Indeed, menadione even in the presence of ATP did not promote significant mitochondrial hyperpolarization (Fig. 8*E*). These observations also indicate that mitochondrial hyperpolarization is essential for NLRP3 inflammasome activation upon mitochondrial dysfunction.

Potassium Efflux and Deubiquitination of NLRP3 Are Implicated in Rotenone/ATP-mediated Caspase-1 Activation—Extracellular ATP stimulates the efflux of intracellular potassium through its binding to the P2X7 receptor (30). Potassium efflux is a crucial cellular event for NLRP3 inflammasome assembly and activation (19), although its underlying mechanism remains poorly understood. To examine whether potassium efflux is associated with the caspase-1 activation by rotenone/ATP stimulation, cells were treated with glibenclamide or a high concentration of extracellular KCl to block potassium

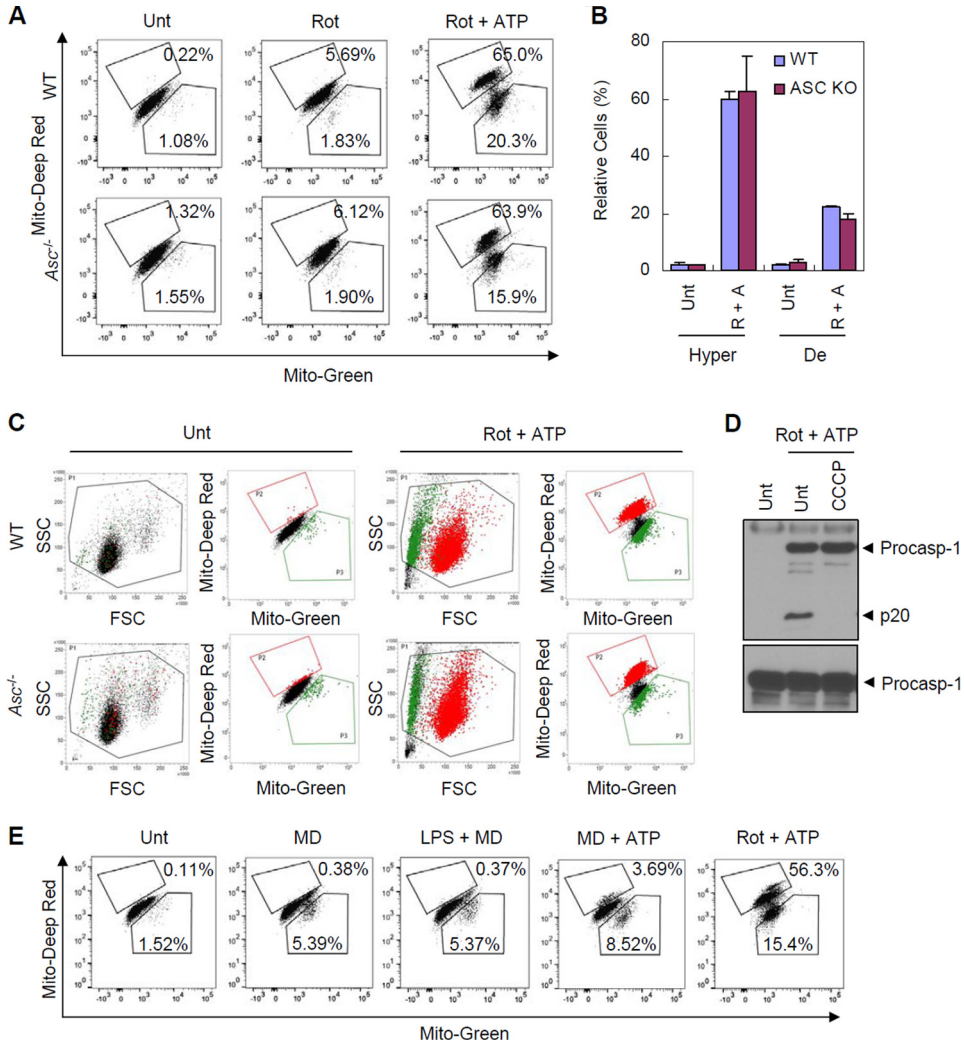

FIGURE 8. **Rotenone/ATP-mediated alterations in mitochondrial membrane potential are independent of inflammasome activation.** *A–C*, immortalized WT or ASC-deficient BMDMs were treated with rotenone (*Rot*), followed by treatment with ATP (2.5 mM, 15 min). Cells were costained with MitoTracker Deep Red and MitoTracker Green and analyzed by a flow cytometer. Relative cell populations in the *top left* (Hyper) and the *bottom right panels* (*De*) were plotted as in *B* ($n = 4$). Cells were also analyzed by forward scatter (*FSC*) and side scatter (*SSC*) as in Fig. 7*C*. *D*, mouse BMDMs were treated with rotenone in the presence of CCCP (5 μM), followed by treatment with ATP. Culture supernatants (*top*) or soluble lysates (*bottom*) were immunoblotted. *E*, mouse BMDMs were treated with menadione (*MD*) (20 μM, 2 h) in the presence of LPS, as indicated, or rotenone (5 μM, 2 h), followed by ATP treatments. Cells were costained with MitoTracker Deep Red and MitoTracker Green and analyzed by a flow cytometer. *Unt*, untreated. *Error bars*, S.E.

efflux. Consequently, the inhibition of potassium efflux by glibenclamide or extracellular KCl largely abolished rotenone/ATP-mediated caspase-1 activation (Fig. 9, *A* and *B*), demonstrating that potassium efflux is potentially involved in mitochondrial dysfunction-mediated caspase-1 activation. By contrast, the inhibition of potassium efflux resulted in a negligible or slight reduction in high-grade mROS production and mitochondrial hyperpolarization induced by rotenone/ATP stimulation (Fig. 9*C*). These findings suggest that potassium efflux might be the downstream event of mROS production and hyperpolarization.

Although it is still unclear how NLRP3 is activated upon classical priming and signal 2 stimulations, several mechanisms have recently been proposed, including ERK phosphorylation and deubiquitination of NLRP3. For instance, LPS priming promotes the activation of ERK signaling, which in turn is required for NLRP3 activation (8). In addition, deubiquitination of NLRP3 triggered either by LPS priming or ATP stimulation is also required to activate NLRP3 (6, 7). To examine whether these molecular events are crucial for rotenone/ATP-mediated caspase-1 activation, we used well known selective inhibitors, such as U0126, to inhibit MEK1/2-dependent ERK phosphorylation, and we used PR619 to inhibit general deubiquitinating enzymes. Inhibition of ERK signaling by U0126 attenuated LPS/ATP-mediated caspase-1 activation but did not impair rotenone/ATP-promoted caspase-1 activation (Fig. 9*D*). However, deubiquitinase inhibitor PR619 completely abolished rotenone/ATP-triggered caspase-1 activation (Fig. 9*D*). This observation suggests that rotenone/ATP stimulation may mediate deubiquitination of NLRP3 for its activation.

Discussion

Mitochondrial dysfunctions caused by a genetic mutation or defective function of mitochondrial proteins have been implicated in numerous neurodegenerative or metabolic diseases (14). Many therapeutic drugs are also able to induce mitochon-

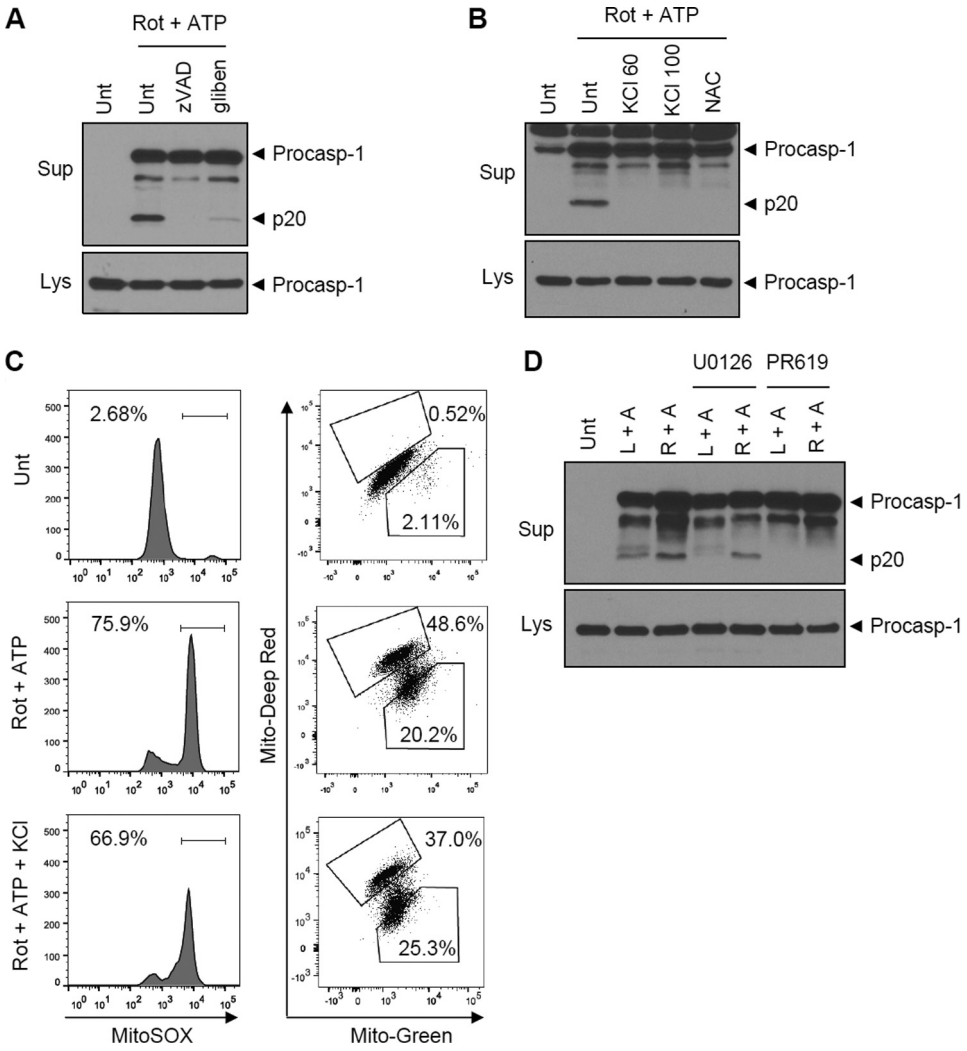

FIGURE 9. **Potassium efflux and NLRP3 deubiquitination are implicated in rotenone/ATP-mediated caspase-1 activation.** *A*, mouse BMDMs were pretreated with Z-VAD (20 μM) or glibenclamide (*gliben*) (50 μM) for 30 min and then treated with rotenone (*Rot*) (5 μM, 3 h), followed by treatment with ATP. *B*, mouse BMDMs were treated with rotenone in the presence of NAC (20 mM), followed by treatment with KCl (60 or 100 mM, 15 min before ATP) and ATP (final 30 min) treatment, as indicated. *C*, mouse BMDMs were treated with rotenone, followed by treatment with KCl (100 mM, 15 min before ATP) and ATP (final 20 min). Cells were analyzed by a flow cytometer after staining with MitoSOX (*left*) or costaining with MitoTracker Deep Red and MitoTracker Green (*right*). *D*, mouse BMDMs were treated with rotenone or LPS in the presence of U0126 (10 μM) or PR619 (5 μM), followed by ATP (30-min) treatment, as indicated. *A*, *B*, and *D*, culture supernatants (*Sup*) or soluble lysates (*Lys*) were immunoblotted. *Unt*, untreated.

drial dysfunction as an off-target effect, possibly causing or aggravating the disease state. In particular, dysfunction of mitochondrial electron transport complex I has been regarded as a main contributor to Leber hereditary optic neuropathy, Leigh syndrome, and Parkinson disease (14, 15, 31). Supporting these findings, the inhibition of mitochondrial complex I by rotenone or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine has been used to induce Parkinson disease in animal models (18, 32). Given the important role played by abnormal mitochondrial ETC in degenerative diseases, our data present further evidence in support of the argument that the impairment of mitochondrial complex I may promote NLRP3 inflammasome activation in the presence of extracellular ATP, which is commonly derived from adjacent injured cells.

Despite growing interest in mitochondrial regulation of the inflammasome pathway, the limited earlier studies were controversial in terms of the effect of mitochondrial dysfunction-inducing chemicals, such as rotenone or CCCP, on NLRP3 inflammasome activation (10, 13, 19, 20). Our results clearly indicate that rotenone, but not CCCP, may trigger NLRP3 inflammasome priming only with ATP costimulation in BMDMs. This finding emphasizes that not all mitochondrial-damaging stimulations contribute to the activation of the NLRP3 inflammasome.

Previous studies focused on mROS production by rotenone-mediated complex I inhibition in order to explain the toxic effect of rotenone (16, 17). Indeed, rotenone treatment caused low-grade mROS production (Fig. 4*A*), but the latter was not sufficient to activate the NLRP3 inflammasome (Figs. 1*A* and 3*D*). Of particular interest, high-grade mROS production was remarkably elevated in rotenone/ATP stimulation but not in rotenone/nigericin, LPS/nigericin, or CCCP stimulation. Further supporting these findings, the antioxidant NAC abolished rotenone/ATP-triggered caspase-1 activation. These data suggest that aberrant production of high-grade mROS, but not weak mROS, is critical for rotenone/ATP-mediated NLRP3

inflammasome activation. However, neither ATP nor menadione/ATP stimulation activates NLRP3 inflammasome despite their ability to produce high-grade mROS. In addition, ATP clearly disrupted mitochondrial cristae structure. These data suggest that high-grade mROS induces a mitochondrial impairment but is not sufficient to trigger NLRP3 inflammasome activation.

Notably, rotenone/ATP stimulation caused alterations in mitochondrial membrane potential. The importance of mitochondrial membrane potential in the NLRP3 inflammasome has not been fully explored. Several previous studies have proposed that CCCP or classical NLRP3-activating stimulations cause mitochondrial depolarization, which may lead to NLRP3 inflammasome activation (10, 12). However, our data indicate that rotenone/ATP-mediated mitochondrial depolarization may be the indication of mitochondrial damage in an inflammasome-independent manner rather than an NLRP3-activating factor. Supporting this hypothesis, mitochondrial depolarization by CCCP completely abolished rotenone/ATP-mediated caspase-1 activation.

Instead, our data suggest that mitochondrial hyperpolarization by rotenone/ATP is potentially implicated in NLRP3 inflammasome activation. The failure of ATP or menadione/ATP to activate caspase-1 may stem from a defect in promoting the hyperpolarization of mitochondria despite their robust production of high-grade mROS. Therefore, we propose that mitochondrial hyperpolarization is needed to activate NLRP3 inflammasome activation upon mitochondrial dysfunction. In accordance with our results, previous studies have proposed that intact mitochondrial membrane potential is required for type 1 interferon production and NLRP3 inflammasome activation in response to RNA virus infection (20, 29). Furthermore, mitochondrial hyperpolarization triggered by the deficiency of UCP-2 (uncoupling protein-2) or oxygen-glucose deprivation exacerbates neuronal damage (33, 34). The molecular mechanism by which mitochondrial hyperpolarization may trigger NLRP3 activation remains poorly understood. Further studies will therefore be needed to elucidate the molecular details involved in this phenomenon.

A previous study has shown that mROS mediates the deubiquitination of NLRP3, which is an important priming step for NLRP3 activation (6). Given that deubiquitination is also implicated in rotenone/ATP-mediated caspase-1 activation (Fig. 9*D*), high-grade mROS may promote NLRP3 inflammasome activation through its deubiquitinating potential in our experimental system. By contrast, mtDNA release appears to result from inflammasome activation upon rotenone/ATP stimulation. This result also emphasizes the fact that indications of mitochondrial damage are not only a priming signal for NLRP3 inflammasome activation but also the consequence of caspase-1 activation.

In conclusion, the impairment of mitochondrial ETC complex I by rotenone increases the sensitivity of the assembly and activation of NLRP3 inflammasome in response to extracellular ATP commonly originating from injured cells or tissues. Robust high-grade mROS production as well as mitochondrial hyperpolarization might mediate the selective activation of NLRP3 inflammasome. Our results thus suggest that a specific inhibition of these mitochondrial alterations may be used to alleviate aberrant NLRP3 inflammasome toxicity.

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